

LEUKOCYTE SUBPOPULATIONS ELICITED BY A NONTUMORIGENIC VARIANT OF B16 MELANOMA: THEIR ROLE IN DIRECT REJECTION OF THE MELANOMA AND IN PREVENTION OF TUMORIGENESIS IN WINN ASSAYS

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A variant of the B16 melanoma, clone C₃471, which is nontumorigenic in immunocompetent mice, is capable of immunizing such mice against the parental, tumorigenic clone B₅59 (1, 2). Clone B₅59 is not immunogenic and does not induce concomitant tumor immunity (3). The immunogenic C₃471 variant was derived from B₅59 cells by continuous growth in the thymidine analog, 5-bromodeoxyuridine (BrdUrd).¹ C₃471 cells have undergone numerous phenotypic changes, including loss of tumorigenicity, pigmentation and plasminogen activator activity, as well as induction of endogenous retrovirus (1, 4–6). Other investigators have also produced immunogenic alterations of tumor cells by xenogenization with viruses (7–9), haptens (10, 11), mutagens (12–15), and with antineoplastic drugs (16–19).

The inability of C₃471 cells to form tumors reflects alteration(s) in interactions of these cells with the host's immune system, rather than an intrinsic loss of tumorigenic potential of C₃471 cells (20). Immunocompetent mice mount an inflammatory response to C₃471 cells within 24 h after inoculation, and inhibit tumor formation by these cells (2, 21). Mice immunologically compromised by treatment with anti-mouse thymocyte serum (ATS) or X irradiation support tumor formation by C₃471 cells.

In the experiments reported here, we have mixed B₅59 cells with peritoneal cells from C₃471 cell-immunized mice and inoculated the mixtures into normal and immunosuppressed mice (Winn-type assay) to analyze leukocyte populations responsible for immunity to B₅59 melanomas. Our results show that normal mice do not develop tumors when inoculated with a mixture of B₅59 cells and peritoneal cells

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¹ *Abbreviations used in this paper:* ATS, rabbit anti-mouse thymocyte serum; BCG, bacillus Calmette-Guerin; BrdUrd, 5-bromodeoxyuridine; FBS, fetal bovine serum; IgG-GRBC, IgG-coated, glutaraldehyde-fixed sheep erythrocytes; MLP, mean latent period; NRS, normal rabbit serum; PBS, phosphate-buffered saline without Ca⁺⁺ or Mg⁺⁺; PEC, peritoneal exudate cells (leukocytes).

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from C₃471 cell-immunized mice, and that a high percentage of these mice retain long-lasting immunity to B₅59 cells. Thy-1.2⁺ peritoneal lymphocytes, or peritoneal macrophages from C₃471 cell-immunized mice each protect normal mice against melanoma formation when coinoculated with B₅59 cells. Thy-1.2⁺ lymphocytes are required for both the generation and maintenance of tumoricidal macrophages in this system. Significantly, tumoricidal macrophages from C₃471-immune mice do not protect antithymocyte serum treated recipient mice against B₅59 melanomas, indicating that recipient thymus-dependent leukocytes are required to effect tumor eradication.

Materials and Methods

Cells. The derivation and maintenance of the melanotic tumorigenic B₅59 clone and the amelanotic, nontumorigenic C₃471 subclone of the B16 melanoma have been described previously (6, 22). Briefly, cells were maintained in monolayer culture in Eagle's minimal essential medium (MEM) (Grand Island Biological Co., Grand Island, NY) supplemented with 7.5% heat-inactivated fetal bovine serum (FBS) (M. A. Bioproducts, Walkersville, MD), 50 µg/ml gentamycin (Schering Corp., Kenilworth, NJ), 50 µg/ml aureomycin (Lederle Laboratories, Wayne, NJ), and 2.5 µg/ml fungizone (E. R. Squibb & Sons, Inc., Princeton, NJ). Cultures were incubated at 37°C in a humidified atmosphere containing 5% CO₂. Cells of the C₃471 subclone were grown continuously in the presence of 1 µg/ml BrdUrd added to the maintenance medium. Semiconfluent monolayers were dispersed using 0.0125% trypsin (Worthington Biochemical Corp., Freehold, NJ) with 2.6 mM EDTA. Total cell counts were determined with a Coulter counter and cell viability measured by trypan blue dye exclusion using a hemocytometer. Cells were checked periodically for mycoplasma using the Hoechst stain method (23); no mycoplasma were detected.

Mice. Female C57BL/6 mice, 5–7 wk of age, were obtained from the Mammalian Genetics Animal Production Section, Division of Cancer Treatment, National Cancer Institute at the Charles River Breeding Laboratories, Inc., Wilmington, MA.

Immunization. C₃471 cells used for immunizing mice were dispersed with trypsin, washed with serum-free MEM, and resuspended at 5×10^6 viable cells/ml MEM for inoculation. All mice used as donors of immune leukocytes for Winn assays were immunized by three intraperitoneal inoculations of 10^6 viable cells at weekly intervals.

To determine the effect of immunization route on the capacity of the animal to reject a tumor challenge, a similar immunization schedule was followed using subcutaneous injection of C₃471 cells into the flank, followed by inoculation of 2×10^6 viable B₅59 cells (the minimal number required to yield tumors in 100% of mice injected) 7 d after the last immunization, either intraperitoneally or subcutaneously, into the opposite flank. Mice receiving subcutaneous challenge with B₅59 cells were monitored for tumor formation by examination of the inoculation site for palpable melanoma nodules. Tumor development in the peritoneal cavity was monitored initially by swelling of the abdomen, and confirmed by dissection 40 d after challenge. Intraperitoneal B₅59 tumors are easily visualized macroscopically as pigmented masses or extensive "peppering" of the mesentery.

Irradiation. Mice were exposed to 650 rad from a 2,400 Ci caesium source gamma cell (located at Memorial Sloan-Kettering Cancer Center, New York), and were maintained in sterile bedding and provided with sterile drinking water acidified to pH 2.0 with 1 N HCl.

In Vivo Depletion of T Lymphocytes. Naïve or C₃471 cell-immunized mice were inoculated intraperitoneally with 0.2 ml rabbit ATS (M. A. Bioproducts) on days 6, 5, 3, 2, and 1 before use in an experiment. On day 0, mice were either inoculated with 2×10^5 B₅59 tumor cells, inoculated with mixtures of peritoneal cells and B₅59 cells for Winn assays, or killed for harvest of peritoneal exudate cells (PEC). Wright-Giemsa staining of cytocentrifuged PEC from ATS-treated C₃471-immunized mice revealed 65% macrophages, 2% lymphocytes, 16% neutrophils, 17% eosinophils, and 0.5% mast cells.

PEC. PEC were harvested by lavage of the peritoneal cavities of naïve or C₃471 cell-immunized mice with balanced salt solution containing 1 mM EDTA and 1 mM Hepes

according to a modification of the procedures of Cohn and Benson (24) as described by Michl et al. (25). Immune PEC were collected 7 d after the last inoculation of C₃471 cells. Lysis of contaminating erythrocytes was done routinely using hypotonic salt solutions. The leukocytes were then centrifuged (600 g/12 min/4°C) and resuspended in MEM without serum. Cells were enumerated using a hemocytometer and viability determined by trypan blue dye exclusion. Wright-Giemsa staining of cytocentrifuged PEC from C₃471 cell-immunized mice revealed 82% mononuclear leukocytes (macrophages and lymphocytes), 6% eosinophils, 3% neutrophils, and 3% mast cells.

Fractionation of PEC Subpopulations

GLASS ADHERENT AND NONADHERENT. Subpopulations of C₃471-immune PEC were separated on the basis of adherence to glass. Glass-adherent leukocytes were separated from nonadherent cells by incubation of $3-4 \times 10^7$ PEC in 100-mm sterile glass petri dishes with MEM supplemented with 15-20% FBS for 2-2.5 h at 37°C in a humidified atmosphere containing 5% CO₂. Approximately 95% of PEC that adhered to glass in a 6-h incubation adhered in the first 2.5 h after plating. An average of 47% ($\pm 5\%$) C₃471-immune PEC are glass adherent within 2.5 h. Nonadherent PEC were transferred to tubes on ice. The adherent PEC monolayers were washed three times each with 5 ml of MEM at 25°C. Adherent PEC were washed twice more with ice-cold PBS containing 1 mM EDTA and 1 mM Hepes (Sigma Chemical Co., St. Louis, MO). After discarding these fluids, adherent cell monolayers were incubated with cold PBS containing EDTA-Hepes for 5-10 min at 4°C. Adherent PEC were then gently scraped using a sterile rubber policeman and transferred to tubes on ice. Both adherent and nonadherent PEC were pelleted (600 g/12 min/4°C) and resuspended in MEM without serum for counting and viability determinations as above. Wright-Giemsa staining of cytocentrifuged preparations of glass-adherent immune PEC revealed 97% mononuclear leukocytes, 1% mast cells, and 1% neutrophils. Over 95% of these mononuclear leukocytes were macrophages as measured by their capacity to bind and/or ingest glutaraldehyde fixed erythrocytes coated with IgG (Table I). Similar staining of leukocytes in the fraction not adherent to glass revealed 90% mononuclear leukocytes, 3% mast cells, and 5% eosinophils. Less than 7% of these leukocytes ingested any IgG-coated erythrocytes.

FRACTIONATION ON NYLON WOOL. Nonadherent PEC were further purified for enrichment of T lymphocytes by fractionation on nylon wool columns based on the method of Julius et al. (26). Nylon wool from Leuko-Pak Leukocyte Filters (Fenwal Laboratories, Deerfield, IL) was soaked in 0.2 N HCl, boiled in distilled water, and autoclaved. Before addition of leukocytes, the columns were washed and preincubated with PBS containing 5% heat-inactivated FBS prewarmed at 37°C. After addition of $0.5-1.5 \times 10^8$ viable glass-nonadherent PEC suspended in prewarmed PBS + 5% FBS, columns were incubated sealed for 1 h at 37°C. PEC not adhering to the nylon wool were eluted dropwise with at least two column volumes of warm PBS + 5% FBS into centrifuge tubes, and cell number and viabilities were determined. Wright-Giemsa staining of cytocentrifuged nylon wool eluate cells revealed a virtually pure (98%) population of lymphocytes.

Activation of Adherent PEC In Vitro. PEC harvested from naïve C57BL/6 mice or from ATS-treated C₃471-immunized mice were plated on glass petri dishes to separate glass adherent and nonadherent fractions as above. Adherent cell monolayers were washed to remove nonadherent leukocytes, and PEC in the glass-nonadherent or nylon wool-purified fractions from either unimmunized normal C57BL/6 mice or from C₃471-immunized C57BL/6 mice were incubated

TABLE I
Binding and Phagocytosis of IgG-Coated Erythrocytes by Glass-Adherent PEC

	Ingested or bound particle	Phagocytic index*	Percent control	Binding index*	Percent control
Resident	IgG-GRBC	442.5 (92)‡	100	937.2 (96)‡	100
C ₃ 471-immune	IgG-GRBC	738.7 (95)	165	1324.8 (96)	141

* Phagocytic and binding indices were determined as described previously (3).

‡ Percent macrophages binding or ingesting IgG-GRBC.

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with the glass-adherent cells for ~18 h in MEM containing 10% FBS. At the end of incubation, nonadherent PEC were removed and adherent cells were washed with MEM, PBS, and then PBS containing 1 mM EDTA and 1 mM Hepes, to minimize contamination with nonadherent leukocytes. PEC still adherent to the glass petri dishes were then harvested by scraping, as above, counted and coinjected with B₅59 cells in Winn assays.

Antisera. Immune lysis of T lymphocytes from unfractionated and fractionated PEC was carried out using either rabbit ATS having a cytotoxic titer of 1:3,200, or monoclonal mouse anti-Thy-1.2 (gift of Dr. U. Hämmerling, Memorial Sloan-Kettering Cancer Center) having a titer of 1:10,000. 4–6-wk-old rabbit complement obtained from Pel-Freez Biological, Inc. (Rogers, AZ) was used at a 1:4 dilution to effect lysis. PEC pellets containing $0.25\text{--}2.5 \times 10^7$ viable cells were resuspended in a total volume of no more than 200 μl of either rabbit ATS or anti-Thy-1.2 in PBS (with Ca^{++} and Mg^{++}), incubated on ice for 30–40 min, washed in PBS, resuspended in 200 μl PBS containing rabbit complement at 1:4 dilution, and incubated for 20 min at 37°C in a CO₂ incubator. Cell viability was estimated by trypan blue dye exclusion.

Tumor Neutralization Assay. A modification of the adoptive tumor neutralization assay described by Winn (27) was used to evaluate the effectiveness of immune peritoneal cell populations in preventing tumor formation by B₅59 melanoma cells. Unfractionated PEC, harvested from naïve or immune mice as described above, were washed in PBS and resuspended in MEM without serum. Subpopulations of immune PEC were similarly prepared after fractionation. PEC were mixed with freshly harvested B₅59 cells to give the appropriate PC/B₅59 cell ratio in a final volume of 0.2 ml. This cell mixture was inoculated subcutaneously into the flank of C57BL/6 mice and animals were checked two to three times weekly for appearance of palpable tumor nodules. Tumor formation is reported for all animals used in each experiment.

In Vitro Cytotoxicity Assay. A colony assay (V. H. Freedman, G. Kaplan, C. S. Copeland, and S. C. Silverstein, manuscript in preparation) was used to measure cytotoxicity. Briefly, tumor cells were plated in 16-mm Costar wells (Costar, Data Packaging, Cambridge, MA), and overlaid with PEC from immunized mice. After 24 h incubation, the tumor cells were trypsinized, diluted, and plated in 60-mm petri dishes. Colonies were counted after 7 d. The percent B₅59 cells killed was calculated as number of colonies in experimental sample divided by the number of colonies in control sample times 100.

Results

Effect of Immunization Route on Rejection of B₅59 Melanoma. We compared the efficacy of subcutaneous vs. intraperitoneal immunization with C₃471 cells in protecting C57BL/6 mice against challenge with various numbers of malignant B₅59 melanoma cells. Fig. 1 shows that at all tumor cell doses tested, mice immunized intraperitoneally were more resistant to intraperitoneal challenge with B₅59 cells than were mice immunized subcutaneously. Subcutaneous immunization provided good protection against subcutaneous or intraperitoneal challenge only at the lowest tumorigenic dose (2×10^5 B₅59 cells). B₅59 tumors developed in 100% of unimmunized animals. As will become evident, the peritoneal cavities of mice immunized with C₃471 cells contain tumoricidal leukocytes. Fig. 1 shows that repeated intraperitoneal immunization with C₃471 cells was the most efficient method of eliciting these leukocytes. Therefore, all subsequent studies were performed using peritoneal leukocytes elicited by intraperitoneal immunization.

Effects of Immunosuppression on Rejection of B₅59 Melanoma by C₃471 Cell-immunized Mice. C57BL/6 mice treated with ATS before inoculation with C₃471 cells develop progressive amelanotic C₃471 tumors (20, 21, 28). To determine whether ATS alters established immunity to the malignant parental melanoma (clone B₅59), mice immunized with C₃471 cells were treated with ATS before subcutaneous challenge with B₅59 cells. All of these mice developed melanotic tumors. In contrast, all of the immunized control mice that received normal rabbit serum (NRS) were protected

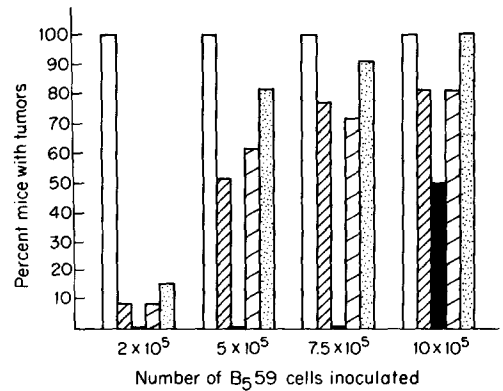


FIG. 1. Effect of immunization route on rejection of subcutaneous vs. intraperitoneal challenge with B₅₅₉ melanoma cells. C57BL/6 mice were immunized by either three subcutaneous (s.c.) or intraperitoneal (i.p.) inoculations of 10⁶ C₃₄₇₁ cells, each 7 d apart. Immunized mice were challenged by either subcutaneous or intraperitoneal injection of the indicated numbers of B₅₅₉ melanoma cells, 7 d after the last C₃₄₇₁ cell inoculation. □, Unimmunized C57BL/6 control mice challenged either i.p. or s.c.; ▨, s.c. immunized mice challenge i.p.; ■, i.p. immunized mice challenge i.p.; ▤, s.c. immunized mice challenged s.c.; ▥, i.p. immunized mice challenge s.c. Mice were monitored for tumor formation as described in Materials and Methods. Data represent averages from four experiments.

TABLE II
Lack of Protection Against B₅₅₉ Melanoma in ATS-treated C₃₄₇₁-immunized Mice*

Mice injected subcutaneously with 2 × 10 ⁵ B ₅₅₉ cells	Mice with tumors/mice inoculated	MLP ± SD
C ₃₄₇₁ -immunized	0/8	—
ATS-treated, C ₃₄₇₁ -immunized	12/12	9.9 ± 2.1
NRS-treated, C ₃₄₇₁ -immunized	0/4	—
Unimmunized controls	12/12	12.9 ± 7.6

* Rabbit ATS or normal rabbit serum injected intraperitoneally into C57BL/6 mice immunized with C₃₄₇₁ cells (Materials and Methods).

against tumor formation (Table II). These results indicate that established immunity can be abrogated by ATS treatment; they suggest that ATS-sensitive leukocytes are involved in both the afferent and efferent pathways of C₃₄₇₁ cell-elicited host defense.

To evaluate the radiosensitivity of the cells that mediate B₅₅₉ tumor cell rejection, C57BL/6 mice were immunized with C₃₄₇₁ cells and exposed to 650 rad of X irradiation 24 h before inoculation with B₅₅₉ cells. These mice were unable to reject the B₅₅₉ cells (data not shown), indicating that radiosensitive effector cell(s) are required for destruction of B₅₅₉ cells in C₃₄₇₁ cell-immune mice.

Characterization of the Leukocytes Elicited by C₃₄₇₁ Cells. To confirm that leukocytes from C₃₄₇₁ cell-immunized mice are responsible for the rejection of B₅₅₉ cells, we mixed varying numbers of C₃₄₇₁-immune peritoneal leukocytes with 2 × 10⁵ B₅₅₉ cells and inoculated them subcutaneously into naïve C57BL/6 mice. C₃₄₇₁ cell-immune PEC markedly inhibited tumorigenesis by B₅₅₉ cells, completely abrogating tumor formation at PEC/melanoma cell ratios of 30:1 or greater, and inhibiting tumor formation in 90% of mice inoculated with a 25:1 ratio. The latter is therefore the ratio of PEC/melanoma cells used for all subsequent Winn assays. In those few

animals that did develop tumors, the mean latent period (MLP) for development of palpable subcutaneous nodules increased progressively as the ratio of PEC/melanoma cells decreased (Fig. 2). As expected, at all ratios tested (10:1 to 40:1), PEC from unimmunized C57BL/6 mice had no inhibitory effect on tumor formation by B₅59 cells (data not shown).

To identify the kinds of leukocytes responsible for the tumoricidal effects of C₃471-immune PEC, we carried out tumor neutralization assays using glass-adherent (macrophage-enriched) and glass-nonadherent (lymphocyte-enriched) subpopulations. 5×10^6 leukocytes from each subpopulation were as effective in protecting mice against B₅59 melanoma as unfractionated immune PEC (Table III). For those few mice that developed B₅59 tumors, the MLP was nearly doubled. Similar protection was obtained when mice were inoculated with a mixture of 2.5×10^6 each of glass-

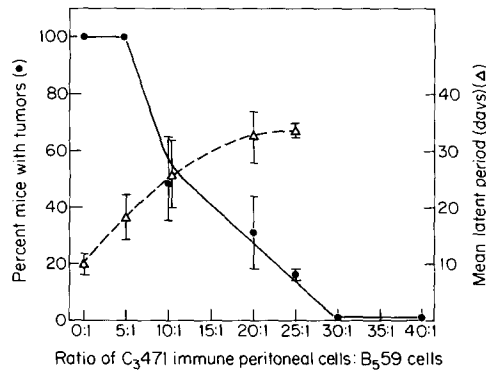


FIG. 2. Inhibition of B₅59 tumor formation by varying numbers of C₃471-immune PEC. Peritoneal leukocytes, from mice immunized by three successive weekly intraperitoneal inoculations of 10^6 viable C₃471 cells, were co-injected subcutaneously, in varying ratios with 2×10^5 viable B₅59 melanoma cells into immunocompetent naïve C57BL/6 mice. Mice were monitored for B₅59 tumor formation by palpation of injection sites two to three times weekly. Data represent averages from seven experiments.

TABLE III
PEC from C₃471 Cell-immunized Mice Inhibit Tumor Formation by B₅59 Cells

C ₃ 471-immune PEC*	Mice with tumors/ mice inoculated	MLP ± SD
Unfractionated	5/26	20.6 ± 15.5
Glass adherent	4/30	21.0 ± 9.9
Glass nonadherent	6/33	21.3 ± 6.1
Glass adherent + glass nonadherent‡	5/18	18.0 ± 9.8
Controls§		
Unfractionated nor- mal C57BL/6	29/32	13.7 ± 8.4
PEC + B ₅ 59 cells	11/12	11.6 ± 4.2

* PEC from C₃471-immunized mice inoculated in a mixture with B₅59 melanoma cells at a 25:1 ratio using 2×10^5 viable melanoma cells.
‡ Glass adherent and nonadherent immune PEC mixed together at a 1:1 ratio after separation on glass as described in Materials and Methods.
§ 2×10^5 B₅59 cells inoculated either alone or together with 5×10^6 PEC from naïve mice.

adherent and nonadherent fractions together with B₅59 cells (Table III). These findings differ from those we reported previously using glass-adherent and nonadherent fractions of PEC elicited by immunization with heat-killed bacillus Calmette-Guerin (BCG) (3). In those experiments, only the glass-adherent leukocytes were able to confer protection against B₅59 tumor formation.

To characterize the cells in the nonadherent PEC population that were responsible for rejection of B₅59 melanomas, nonadherent PEC from C₃471 immune mice were treated with anti-thymocyte serum or anti-Thy-1.2 and complement and then co-injected with B₅59 cells into naïve mice. This treatment completely abrogated the capacity of immune nonadherent PEC to protect mice against B₅59 melanoma (Table IV). To confirm that the T cells alone were capable of inhibiting melanoma formation, immune T cells were purified by passage of C₃471-immune nonadherent PEC over a nylon wool column. Mice inoculated with a mixture of these T cells and B₅59 melanoma cells were protected against B₅59 melanomas; this protection was abolished by treatment of the immune T cells with anti-thymocyte serum or anti-Thy-1.2 and complement (Table IV). In contrast, treatment of C₃471 immune glass-adherent PEC with ATS or anti-Thy-1.2 and complement had no effect on the capacity of these leukocytes to inhibit B₅59 tumorigenesis (Table IV). These results indicate macrophages and thymus-derived lymphocytes are responsible for the *in vivo* tumor inhibitory effects of the corresponding glass-adherent and nonadherent C₃471 cell-immune PEC fractions; and that macrophages from C₃471 immunized mice retain their tumoricidal activity in the absence of immune donor T cells.

Participation of Recipient ATS-sensitive Leukocytes in Rejection of B₅59 Melanoma Cells. ATS-sensitive lymphocytes can be removed from either unfractionated or glass-adherent immune PEC without impairing their capacity to cause rejection of B₅59 cells in Winn assays in normal mice (Table IV). Therefore, both unseparated and adherent PEC should yield a high degree of protection if recipient T cells do not

TABLE IV
Effects of In Vitro Treatment of C₃471-immune PEC With Anti-Thy-1.2 or Rabbit ATS on Their Tumor Inhibitory Properties

C ₃ 471-immune PEC subpopulation*	Treatment					
	C' Alone‡		Anti-Thy-1.2§ + C'		ATS + C'	
	Percent lysis	Mice with tumors/mice inoculated¶	Percent lysis	Mice with tumors/mice inoculated¶	Percent lysis	Mice with tumors/mice inoculated¶
Unfractionated	1.6	0/6	18.8	1/6	20.7	0/8
Glass adherent	0.9	0/4	3.5	0/5	4.2	1/5
Glass nonadherent	1.1	0/4	47.1	6/6	49.4	4/4
Nylon wool nonadherent	0.9	1/21	94.5	8/8	95.3	4/4

* PEC were obtained and fractionated as described in Materials and Methods. The nylon wool-nonadherent cells were obtained by passage of glass-nonadherent cells through nylon wool columns as described in Materials and Methods.

‡ Rabbit complement diluted 1:4.

§ Monoclonal mouse anti-Thy-1.2 diluted 1:200.

|| Rabbit anti-mouse thymocyte serum diluted 1:100.

¶ PEC inoculated with B₅59 melanoma cells at 25:1 ratio using 2×10^5 viable melanoma cells and 5×10^6 PEC.

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participate in the rejection of B₅59 cells in Winn assays. To examine this question, naïve mice were treated with ATS before inoculation with C₃471 cell-immune PEC and B₅59 cells. ATS treatment was suspended 24 h before leukocyte transfer to minimize effects of circulating anti-T cell antibody on immune donor PEC. As expected, the glass-nonadherent fraction was unable to protect against tumor formation in the ATS-treated recipients. Surprisingly, however, neither unfractionated C₃471-immune PEC (containing macrophages and T cells), nor the glass-adherent subfraction (comprised primarily of macrophages), was able to convey protection against B₅59 melanoma upon adoptive transfer into ATS-treated syngeneic mice (Table V).

The inability of the macrophage-enriched fraction to protect ATS-treated recipients against B₅59 melanoma suggested that recipient T cells participate in tumor neutralization by C₃471-immune macrophages. This hypothesis is tenable only if the ATS used in these experiments did not inhibit macrophage recruitment, viability, or tumoricidal effector functions. Two lines of evidence show that it did not. First, mice immunized intraperitoneally with C₃471 cells for 3 successive wk were treated with ATS as described in Materials and Methods. Recovery of total PEC from these mice was invariably nearly twice that from untreated immune mice (7.6×10^6 vs. 4.5×10^6), and Wright-Giemsa-stained cytocentrifuge preparations of these PEC contained 65% macrophages, 2% lymphocytes, 16% neutrophils, and 17% eosinophils. PEC from ATS-treated immune mice contained more macrophages than PEC from immune mice (4.9×10^6 vs. 2.25×10^6). Second, ATS in the presence (Table IV) or absence of complement (Tables VI and VII) did not inhibit the tumoricidal activity of immune macrophages in vitro (Table VI) or in vivo (Tables IV and VII). That the concentration of ATS used exceeded that in the serum of ATS pretreated mice at the time of Winn assay was determined as follows: ATS at a dilution of 1:100 lysed 95% of C₃471-immune nylon wool-purified T cells (Table IV), whereas undiluted serum from mice treated with ATS (as in Table V) plus complement lysed only 30–37% of these T cells (data not shown). No detectable lysis of C₃471-immune macrophages was observed when these cells were incubated with undiluted serum from ATS treated

TABLE V
Treatment of Recipient Mice with ATS Blocks the Capacity of C₃471-Immune PEC to Inhibit B₅59 Melanoma Formation in Winn Assays

C ₃ 471-immune subpopulation*	Untreated recipients‡		ATS-treated recipients§	
	Mice with tumors/mice inoculated	MLP ± SD	Mice with tumors/mice inoculated	MLP ± SD
Unfractionated	0/11	—	14/14	10.4 ± 2.0
Glass adherent	1/6	19.0 ± 0	7/7	14.5 ± 1.7
Glass nonadherent	1/7	26.0 ± 0	7/7	15.7 ± 3.8
B ₅ 59 Controls	11/11	9.4 ± 2.4	12/12	8.3 ± 3.1

* As in Table IV.

‡ Normal C57BL/6 mice.

§ Normal C57BL/6 mice pretreated with rabbit ATS. These mice received five 0.2-cc inoculations of ATS intraperitoneally on days -6, -5, -4, -2, and -1 before subcutaneous inoculation of immune PEC with melanoma cells for Winn assay.

TABLE VI
ATS Does Not Inhibit Killing of B₅59 Melanoma Cells by C₃471-immune Adherent PEC In Vitro

Number of adherent PEC*/well	ATS‡	Percent B ₅ 59 cells viable§
5 × 10 ⁶	—	0.01%
5 × 10 ⁶	+	0.01%
2 × 10 ⁶	—	0.5%
2 × 10 ⁶	+	0.5%
—	—	100%
—	+	100%

* Glass-adherent PEC from C₃471-immunized mice harvested as described in Materials and Methods.

‡ ATS at a 1:100 concentration in MEM with 10% FBS.

§ Percentage of B₅59 melanoma cells killed is based upon the number of colony-forming units of B₅59 cells remaining after 24 h incubation at 37° of a mixture of 1 × 10⁵ B₅59 cells and the indicated number of C₃471-immune PEC. B₅59 cells were harvested and plated; colonies were enumerated as described in Materials and Methods. 100% represents the average number of B₅59 colonies recovered from control wells.

TABLE VII
Co-Injection of ATS With C₃471-immune Macrophages Does Not Inhibit Their Tumoricidal Activity

	Mice with tumors/ mice inoculated	MLP ± SD
C ₃ 471-immune adherent PEC*	0/4	—
C ₃ 471-immune adherent PEC + ATS‡	0/6	—
B ₅ 59 controls	5/6	16.4 ± 3.5

* Each mouse was inoculated subcutaneously with 0.5 ml MEM containing 5 × 10⁶ viable glass-adherent PEC from C₃471-immunized mice and 2 × 10⁵ B₅59 melanoma cells.

‡ Each mouse was inoculated subcutaneously with 0.2 ml MEM containing 5 × 10⁶ viable glass-adherent PEC from C₃471-immunized mice, 2 × 10⁵ B₅59 melanoma cells, and 2 μl ATS. The final ATS concentration was 1:100.

TABLE VIII
*Effect of ATS Treatment In Vivo on Tumoricidal Activity of PEC of C₃471-Immunized Mice**

C ₃ 471-immune subpopulation	Mice with tumors/mice inoculated		
	From untreated immune donors‡	From ATS-treated immune donors§	From NRS-treated immune donors
Unfractionated	2/29	29/30	0/5
Glass adherent	0/10	14/16	0/8
Glass nonadherent	0/12	11/12	0/8
B ₅ 59 Controls¶	35/36		

* 5 × 10⁶ PEC harvested from C₃471-immune mice, prepared as described in Materials and Methods, were inoculated together with 2 × 10⁵ B₅59 cells into naive C57BL/6 mice.

‡ PEC from untreated C₃471-immunized mice as in Table IV.

§ PEC from C₃471-immunized mice as above harvested 24 h after the last inoculation of rabbit ATS. Mice were treated with ATS for 3 d (days -6, -5, and -4) after their last immunization with C₃471 cells, and again on the 2 d (days -2 and -1) before harvest of their PEC as described in Materials and Methods.

|| Mice were treated with NRS following the schedule described above (§).

¶ 2 × 10⁵ B₅59 cells inoculated subcutaneously into naive C57BL/6 mice.

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TABLE IX
Activation of Macrophage Tumoricidal Activity by C₃471-Immune Lymphocytes

Source of glass-adherent PEC used in Winn Assays*	Cells incubated with glass adherent PEC	Mice with tumors/mice inoculated	MLP \pm SD
ATS-treated, C ₃ 471-immunized	Glass nonadherent from normal C57BL/6 mice	7/7	11.4 \pm 3.5
	Nylon wool eluate from normal C57BL/6 mice	5/5	18.3 \pm 7.9
	Glass nonadherent from C ₃ 471-immunized mice	2/21	17.0 \pm 1.4
	Nylon wool eluate from C ₃ 471-immunized mice	1/5	26.0
Normal C57BL/6 mice	Glass nonadherent from normal C57BL/6 mice	3/3	22.3 \pm 9.8
	Nylon wool eluate from normal C57BL/6 mice	4/4	15.8 \pm 5.3
	Glass nonadherent from C ₃ 471-immunized mice	0/5	—
	Nylon wool eluate from C ₃ 471-immunized mice	1/4	18.0
B ₅ 59 cells	None	13/14	12.6 \pm 4.1

* Glass-adherent PEC were harvested from either naïve, untreated C57BL/6 mice or from C₃471-immunized mice injected intraperitoneally with rabbit ATS after the last inoculation of C₃471 cells and before harvest of PEC as in Table VIII and as described in Materials and Methods. The nonadherent cells were incubated with the adherent PEC for 24 h and then removed by washing. The adherent cells were removed from the monolayer, mixed with B₅59 cells, and inoculated into naïve mice at a ratio of 5×10^6 viable adherent cells: 2×10^5 B₅59 cells as described in Materials and Methods.

mice in the presence of complement for 1–3 h at 37°C (data not shown).

ATS-sensitive T Lymphocytes are Required for the Maintenance of Tumoricidal Macrophages in C₃471 Cell-immune Mice. To determine whether T cell functions are required for the maintenance of macrophage cytotoxic effector functions in vivo C₃471 cell-immunized mice were treated with ATS after their last immunization with C₃471 cells. Their PEC were then harvested and co-inoculated together with B₅59 cells into naïve C57BL/6 mice. ATS treatment of the immunized donor mice resulted in abrogation of the tumor protective capacity of their PEC; both adherent and nonadherent PEC were inactive. Normal rabbit serum had no effect (Table VIII). These results suggested that the inhibitory action of ATS on the maintenance of macrophage tumoricidal activity was an indirect one, a consequence of ATS-mediated destruction of T lymphocytes. If so, then addition of C₃471-immune lymphocytes to these macrophages should restore their tumoricidal activity. To test this hypothesis, glass-adherent PEC harvested from ATS treated immune mice, were washed to remove nonadherent leukocytes, and incubated for 18 h with nonadherent or nylon wool-purified leukocytes from C₃471-immunized donors or from nonimmunized C57BL/6 mice. Nonadherent PEC were removed by extensive washing. 92% of the glass-adherent cells ingested IgG-coated glutaraldehyde-fixed sheep erythrocytes, showing that the washing procedure was sufficient to remove virtually all added immune lymphocytes. The macrophages were harvested and injected together with

B₅59 melanoma cells into naïve C57BL/6 mice. Macrophages incubated with either glass nonadherent or nylon wool-purified leukocytes from immunocompetent C₃471-immune mice, conferred a high degree of protection against B₅59 tumor formation to naïve Winn assay recipients (Table IX). Macrophages incubated with either nonadherent or nylon wool-purified cells from unimmunized C57BL/6 mice provided no protection against tumor formation (Table IX). These results confirm that T lymphocytes from C₃471-immune mice are essential for the generation and maintenance of the tumor inhibitory properties of macrophages.

To determine whether lymphocytes from C₃471 cell-immune animals could stimulate macrophage tumoricidal activity in the absence of tumor antigens in the adherent cell population, macrophages from normal, nonimmunized C57BL/6 mice were incubated with glass nonadherent or nylon wool eluate PEC from C₃471-immunized mice. At the end of the incubation, the nonadherent cells were removed by extensive washing. (Over 90% of the remaining adherent leukocytes were macrophages as measured by their ingestion of IgG-coated glutaraldehyde-fixed erythrocytes [IgG-GRBC].) The adherent leukocytes were detached from the plates and coinjected with B₅59 melanoma cells into naïve mice. These adherent cells prevented tumor formation in eight of nine recipients (Table IX). All animals receiving macrophages that had been incubated with glass nonadherent or nylon wool eluate cells from unimmunized mice developed lethal melanomas (Table IX).

Acquisition of Tumor Immunity by Winn Assay Recipients. B₅59 melanoma cells are not immunogenic. B₅59 tumor formation is not prevented by inoculation of mitomycin-treated or radiation-killed B₅59 cells (1, 29), or by repeated inoculation of crude membranes prepared from cultured B₅59 cells (29) into syngeneic mice. Also, animals bearing B₅59 tumors do not exhibit concomitant tumor immunity (3). Efforts to transfer immunity against B₅59 melanoma to naïve syngeneic mice with C₃471-immune leukocytes have been unsuccessful. Neither 10^7 peritoneal nor 10^7 spleen cells from C₃471-immunized mice conferred any protection against B₅59 tumor formation even when administered on multiple occasions, subcutaneously, or intravenously to normal or sublethally irradiated naïve C57BL/6 mice. In these experiments 27 of 28 normal mice and 22 of 23 irradiated mice receiving C₃471-immune leukocytes developed lethal melanomas when challenged with 2×10^5 B₅59 cells. However, when animals that had received 5×10^6 unfractionated C₃471-immune PEC together with the melanoma cells in a Winn assay were tested by a second injection of B₅59 cells 40–60 d later, 12 of 21 animals remained free of tumors 40 d after this second challenge. Thus, a significant degree of systemic immunity to B₅59 melanoma can be adoptively transferred by immune peritoneal leukocytes in the presence of tumor cells.

Discussion

We have examined the mechanism of rejection of clone B₅59 of B16 melanoma by syngeneic C57BL/6 mice immunized with an immunogenic variant (clone C₃471) of the parental melanoma. Immunization with C₃471 cells results in systemic immunity to B₅59 melanoma (1, 2). The protective effect of immunization is most pronounced when immunization and subsequent challenge are within the same tissue compartment (the peritoneal cavity); nonetheless, significant systemic resistance to tumor challenge is observed when the sites of immunization and/or tumor challenge lie outside the peritoneum (Fig. 1). Treatment of naïve mice with ATS before C₃471

inoculation permits the development of C₃471 tumors (20), and established immunity to B₅59 cells can be abrogated by ATS treatment (Tables II and V) or X irradiation of the immune mice. These findings indicate that intact T cell function is required for the establishment and maintenance of immunity to this melanoma. This conclusion was substantiated when leukocyte subpopulations from immune mice were coinjected with B₅59 cells into naïve or ATS-treated recipients. Macrophage or T cell enriched populations prevented tumor growth in naïve mice but not in ATS-treated mice (Table V).

There are two distinct pathways via which immune T cells might act in this system. T cells might function as cytotoxic effector cells or as amplifier and helper cells. We do not know whether C₃471-immune T cells are themselves cytotoxic. Further work is needed to resolve this issue. However, our data do indicate that these T cells act as amplifiers of the immune reaction, promoting the activation of mononuclear phagocytes that are tumoricidal *in vivo* (Table IX). A requirement for cooperation between mononuclear phagocytes and immune T cells is further emphasized by the inability of macrophages from C₃471-immunized mice to prevent tumors in ATS-treated Winn assay recipients (Table V).

Previous work with macrophages elicited by BCG immunization showed that co-injection of BCG-activated macrophages together with B₅59 cells inhibited melanoma formation in normal, X-irradiated and nude mice (3). Thus it was surprising to find that demonstrably tumoricidal macrophages from C₃471-immune mice did not inhibit B₅59 melanoma formation in mice treated with ATS (Table V). Careful examination of the effects of ATS showed that this serum did not inhibit macrophage recruitment *in vivo*, or macrophage viability or tumoricidal activity *in vitro* (Tables IV, VI, and VII). These findings indicate that the inhibitory effect of ATS on macrophage tumoricidal activity *in vivo* is not due to a direct action of this serum on the macrophages. We conclude that T cell functions are required for the full expression of macrophage tumoricidal activity *in vivo*.

In addition to a requirement for T cells and macrophages, other cell types may be involved in the rejection of B₅59 cells by C₃471 cell-immunized mice. Beige mice immunized with C₃471 cells fail to reject B₅59 melanoma (38), suggesting that natural killer and/or other leukocyte subpopulations that are defective in beige mice (39–41) may be required.

Approximately half of the animals that reject B₅59 cells after co-injection of these melanoma cells with immune PEC developed long lasting immunity to B₅59 cells as measured by their ability to reject a challenge with the tumorigenic melanoma cells. In contrast, as we reported previously (3), mice that did not develop tumors after co-injection of BCG-immune PEC with B₅59 cells remained completely susceptible to subsequent challenge with B₅59 cells. Thus, the ability of the mice that received C₃471-immune peritoneal leukocytes and B₅59 melanoma cells to resist a second challenge 40–60 d later appears to be due to stimulatory effects on the host immune system of adoptively transferred immune leukocytes in the presence of antigen (B₅59 cells).

The BrdUrd-induced changes responsible for the immunizing properties of C₃471 cells have not been defined. However, virus production by C₃471 cells seems to play a key role. C57BL/6 mice immunized with nontumorigenic feral mouse cells chronically infected with C₃471 cell-derived retrovirus are protected against challenge with

B₅59 melanoma cells (38).

In summary, three novel findings emerge from this work. First, that an intact T lymphocyte system is needed to elicit tumoricidal macrophages by immunization with the nontumorigenic BrdUrd-grown melanoma variant. Second, that the tumoricidal macrophages elicited are unable to mediate rejection of B₅59 melanoma cells in a Winn assay in the absence of intact recipient T lymphocyte function, further emphasizing a requirement for cooperation of T cells with other leukocyte populations. Third, that a significant proportion of naïve C57BL/6 mice can be immunized against B₅59 melanoma when these tumor cells are co-injected with peritoneal cells from C₃471 cell-immune mice. This last finding suggests one approach to immunization against nonimmunogenic syngeneic tumors.

Summary

The mechanisms by which various leukocyte subpopulations elicited by an immunogenic, nontumorigenic subclone (C₃471) of B16 melanoma caused rejection of the tumorigenic parental melanoma (B₅59), were investigated. Leukocytes from C₃471-immune mice were co-injected with B₅59 tumor cells in Winn assays into normal syngeneic recipients. Tumor formation by B₅59 cells was prevented when C₃471-immune (a) unfractionated peritoneal leukocytes, or (b) glass-adherent peritoneal cells (90% macrophages), or (c) nylon wool purified nonadherent cells (95% Thy-1.2⁺) were used in the Winn assays. If the C₃471-immunized mice were treated with antithymocyte serum before harvest of their peritoneal cells, none of these leukocyte populations were effective in the Winn assay. However, macrophages from these immunologically compromised donors regained their tumoricidal activity after incubation in vitro with T lymphocytes from untreated C₃471-immune donors; similarly, C₃471-immune lymphocytes rendered normal resident peritoneal macrophages tumoricidal in Winn assays. When C₃471-immunized mice were irradiated or treated with antithymocyte serum before direct challenge with B₅59 cells, melanomas developed, thus providing additional evidence for the need for intact T cell function to establish immunity against the melanoma. Furthermore, when Winn assay recipients were treated with antithymocyte serum, neither C₃471-immune macrophages nor T cells were able to prevent tumor formation. These findings indicate that antithymocyte serum-sensitive (Thy-1.2⁺) lymphocytes are necessary both for the generation of tumoricidal leukocytes in C₃471-immunized mice, and for the rejection of B₅59 melanoma by demonstrably tumoricidal macrophages in Winn assay recipients. In addition, long-lasting immunity developed in 50% of the normal mice that had received both C₃471-immune peritoneal cells and B₅59 tumor cells, as manifested by their capacity to reject a second challenge with B₅59 cells 40–60 d later.

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References

1. Silagi, S. 1976. Effects of 5-bromodeoxyuridine on tumorigenicity, immunogenicity, virus production, plasminogen activator, and melanogenesis of mouse melanoma cells. *Int. Rev.*

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- Cytol.* **45**:65.
2. Silagi, S. 1971. Modification of malignancy by 5-bromodeoxyuridine. Studies of reversibility and immunological effects. *In Vitro (Rockville)*. **7**:105.
 3. Freedman, V. H., T. A. Calvelli, S. Silagi, and S. C. Silverstein. 1980. Macrophages elicited with heat-killed *Bacillus Calmette-Guerin* protect C57BL/6 mice against a syngeneic melanoma. *J. Exp. Med.* **152**:657.
 4. Silagi, S., and S. A. Bruce. 1970. Suppression of malignancy and differentiation in melanotic cells. *Proc. Natl. Acad. Sci. U. S. A.* **66**:72.
 5. Christman, J. K., S. Silagi, E. W. Newcomb, S. C. Silverstein, and G. Acs. 1975. Correlated suppression by 5-bromodeoxyuridine of tumorigenicity and plasminogen activator in mouse melanoma cells. *Proc. Natl. Acad. Sci. U. S. A.* **72**:47.
 6. Silagi, A., D. Beju, J. R. Wrathall, and E. deHarven. 1972. Tumorigenicity, immunogenicity and virus production in mouse melanoma cells treated with 5-bromodeoxyuridine. *Proc. Natl. Acad. Sci. U. S. A.* **69**:3443.
 7. Kobayashi, H., F. Sendo, H. Kaji, T. Shirai, H. Saito, H. Takeichi, M. Hosokawa, and T. Kodama. 1970. Inhibition of transplanted rat tumors by immunization with identical tumor cells infected with Friend virus. *J. Natl. Canc. Inst.* **44**:11.
 8. Kobayashi, H., T. Kodama, and E. Gotohada. 1977. Xenogenization of tumor cells. Hokkaido University Medical Library Series, Vol. 9. Sando Printing Co. Ltd., Sapporo, Japan. 3-124.
 9. Kuzamaki, N., E. M. Fenyo, E. Klein, and G. Klein. 1978. Protective effect of murine sarcoma virus-superinfected mouse tumor cells against outgrowth of corresponding noninfected tumor. *Transplantation (Baltimore)*. **26**:304.
 10. Prager, M. D., and F. S. Baechtel. 1973. Methods for modification of cancer cells to enhance their antigenicity. *Methods Cancer Res.* **IX**:339.
 11. Forman, J. 1977. T cell-mediated cytotoxicity against trinitrophenyl-modified cells: effect of glutaraldehyde treatment on the immunogenicity and antigenicity of trinitrophenyl-modified cells. *J. Immunol.* **118**:1755.
 12. Boon, T., and O. Kellermann. 1976. Rejection by syngeneic mice of cell variants obtained by mutagenesis of a malignant teratocarcinoma cell line. *Proc. Natl. Acad. Sci. U. S. A.* **74**:272.
 13. Boon, T., and A. Van Pel. 1978. Teratocarcinoma cell variants rejected by syngeneic mice: Protection of mice immunized with these variants against other variants and against the original malignant cell line. *Proc. Natl. Acad. Sci. U. S. A.* **75**:1519.
 14. Van Pel, A., M. Georlette, and T. Boon. 1979. Tumor cell variants obtained by mutagenesis of a Lewis lung carcinoma cell line: immune rejection by syngeneic mice. *Proc. Natl. Acad. Sci. U. S. A.* **76**:5282.
 15. Uyttenhove, C., J. Van Snick, and T. Boon. 1980. Immunogenic variants obtained by mutagenesis of mouse mastocytoma P815. I. Rejection by syngeneic mice. *J. Exp. Med.* **152**:1175.
 16. Nicolin, A., S. Vadlamudi, and A. Goldin. 1972. Antigenicity of L1210 leukemic sublines induced by drugs. *Cancer Res.* **32**:653.
 17. Belehradsek, J., J. L. Biedler, M. Thonier, and G. Barski. 1974. Actinomycin-D-resistant *in vitro* mouse cell line derived from a methylcholanthrene induced sarcoma: decrease of malignancy and antigenic characteristics. *Int. J. Cancer.* **14**:779.
 18. Nicolin, A., A. Bini, F. DiPadova, and A. Goldin. 1976. Immunologic cross-reactivity of antigen(s) induced by drug treatment in two leukemic sublines. *J. Immunol.* **116**:1347.
 19. Nicolin, A., F. Veronese, O. Marelli, and A. Goldin. 1980. Immunological resistance to L1210 leukemia induced by viable L1210/DTIC cells. *Cancer Immunol. Immunother.* **9**:43.
 20. Silagi, S., E. W. Newcomb, and M. E. Weksler. 1974. Relationship of antigenicity of

- melanoma cells grown in 5-bromodeoxyuridine to reduced tumorigenicity. *Cancer Res.* **34**:100.
21. Newcomb, E. W., S. C. Silverstein, and S. Silagi. 1978. Malignant mouse melanoma cells do not form tumors when mixed with cells of a non-malignant subclone: relationship between plasminogen activator expression by the tumor cells and the host's immune response. *J. Cell Physiol.* **95**:169.
 22. Silagi, S. 1969. Control of pigment production in mouse melanoma cells *in vitro*: evocation and maintenance. *J. Cell Biol.* **43**:263.
 23. Russell, W. C., C. Newman, and D. H. Williamson. 1975. A simple cytochemical technique for demonstration of DNA in cells infected with mycoplasma and viruses. *Nature (Lond.)*. **253**:461.
 24. Cohn, Z. A., and B. Benson. 1965. The differentiation of mononuclear phagocytes: morphology, cytochemistry, and biochemistry. *J. Exp. Med.* **121**:153.
 25. Michl, J., D. J. Ohlbaum, and S. C. Silverstein. 1976. 2-deoxyglucose selectively inhibits Fc and complement receptor mediated phagocytosis in mouse peritoneal macrophages. I. Description of the inhibitory effect. *J. Exp. Med.* **144**:1465.
 26. Julius, M. H., E. Simpson, and L. A. Herzenberg. 1973. A rapid method for the isolation of functional thymus-derived murine lymphocytes. *Eur. J. Immunol.* **3**:645.
 27. Winn, H. J. 1961. Immune mechanisms in homotransplantation. II. Quantitative assay of the immunologic activity of lymphoid cells stimulated by tumor homografts. *J. Immunol.* **86**:228.
 28. Calvelli, T. A., V. H. Freedman, S. C. Silverstein, and S. Silagi. 1981. *In situ* responses to tumorigenic and nontumorigenic melanoma cells by immunosuppressed mice. *J. Cell Biol.* **91**:77a.
 29. Weintraub, S. B., T. A. Calvelli, and S. Silagi. 1981. Immunization of syngeneic mice against malignant melanoma with subcellular fractions of a bromodeoxyuridine-grown variant. *Cancer Immunol. Immunother.* **12**:63.
 30. Howell, S. B., J. H. Dean, E. C. Esber, and L. W. Law. 1974. Cell interactions in adoptive immune rejection of a syngeneic tumor. *Int. J. Cancer.* **14**:662.
 31. Ting, C-C. 1976. Studies of the mechanisms for the induction of *in vivo* tumor immunity. I. Induction of primary and secondary cell-mediated cytotoxic response by adoptive transfer of lymphocytes. *Cell. Immunol.* **27**:71.
 32. Ting, C-C., D. Rodrigues, and T. Igarashi. 1979. Studies of the mechanisms for the induction of *in vivo* tumor immunity. III. Recruitment of host helper cells by donor T cells in adoptive transfer of cell-mediated immunity. *J. Immunol.* **122**:1510.
 33. Fujiwara, H., T. Hamaoka, G. M. Shearer, H. Yamamoto, and W. D. Terry. 1980. The augmentation of *in vitro* and *in vivo* tumor-specific T cell-mediated immunity by amplifier T lymphocytes. *J. Immunol.* **124**:863.
 34. Lohmann-Matthes, M. L., F. G. Zigler, and H. Fischer. 1973. Macrophage cytotoxicity factor: a product of *in vitro* sensitized thymus-dependent cells. *Eur. J. Immunol.* **3**:56.
 35. Erb, P., and M. Feldman. 1975. The role of macrophages in the generation of T helper cells. III. Influence of macrophage-derived factors in helper cell induction. *Eur. J. Immunol.* **5**:759.
 36. Piessens, W. F., W. H. Churchill, Jr., and J. R. David. 1975. Macrophages activated *in vitro* with lymphocyte mediators kill neoplastic but not normal cells. *J. Immunol.* **114**:293.
 37. Ruco, L. P., and M. S. Meltzer. 1977. Macrophage activation for tumor cytotoxicity: induction of tumoricidal macrophages by supernatants of PPD-stimulated Bacillus Calmette-Guerin-immune spleen cell cultures. *J. Immunol.* **119**:889.
 38. Silagi, S., and T. A. Calvelli. 1982. Relationship between rejection of several syngeneic

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- tumors and retrovirus production by 5-bromodeoxyuridine-grown melanoma cells: lack of protection in NK-deficient beige mice. *Cancer Res.* **42**:2562.
39. Roder, J., and A. Duwe. 1979. The beige mutation in the mouse selectively impairs natural killer cell function. *Nature (Lond.)*. **278**:451.
40. Mahoney, K. H., S. S. Morse, and P. S. Morahan. 1980. Macrophage functions in beige (Chediak-Higashi syndrome) mice. *Cancer Res.* **40**:3934.
41. Saxena, R. K., Q. B. Saxena, and W. H. Adler. 1982. Defective T-cell response in beige mutant mice. *Nature (Lond.)*. **495**:240.